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Detection and characterization of anionic polypeptidic fraction binding sites in rat liver plasma membranes and cultured hepatocytes

M. Martigne [1], B. Melin [2], F. Mahlberg [3], N. Domingo [1], F. Chanussot [1], H. Lafont [1] and J.C. Hauton [1]

[1] *INSERM Unité 130, Marseille,* [2] *INSERM Unité 181, Laboratoire de Biologie Cellulaire, Faculté de Médecine St-Antoine, Paris and* [3] *Laboratoire de Nutrition Humaine, Faculté de Médecine Xavier-Bichat, Université Paris 7, Paris (France)*

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The binding of human ^{125}I-labeled 'anionic polypeptidic fraction' (APF) to purified rat liver plasma membranes was studied. The dissociation constant for this binding was 3.0 μg protein/mg membrane protein. Binding was competitively inhibited by unlabeled human APF, but not by human LDL (low density lipoproteins). When unlabeled HDL_3 was added, binding of labeled APF was competitively reduced to a level between that of unlabeled APF and unlabeled LDL. Experiments with cultured rat hepatocytes confirmed those obtained with liver membranes and suggested the presence in rat liver of saturable APF-binding sites which seem to be specific for APF. The physiologic significance of these APF binding sites is discussed in relation to the fate of cholesterol in the liver.

Introduction

It is generally admitted that the main lipid components of most mammal biles, i.e. bile salts, phosphatidylcholine and free cholesterol, are transported as mixed micelles [1]. However, Pattinson [2] demonstrated that cholesterol in native bile is solubilized as a lipoprotein complex in addition to the mixed micelle. Results obtained in our laboratory [3,4] indicated a close association between biliary lipids, an anionic polypeptidic fraction (APF), and fragments of IgA of relative molecular mass 25000. Together these elements form the 'bile lipoprotein complex'. A recent study of the dynamic structure of the human 'bile lipoprotein complex' [5] showed that in native state is probably a discoidal micellar structure with both S_A (annular surface) and S_D (surface discoidal) lipid/water interface. IgA fragments are probably electrostatically associated to the S_D lipid/water interface. APF seems to be hydrophobically bound to the S_A lipid/water interface perhaps on the acyl chain of the external phosphatidylcholine of the mixed disc [5].

Recently APF was isolated from human 'bile lipoprotein complex' by zonal ultracentrifugation [6]. In another study an immunocytochemical method allowed APF to be localized on the brush border of enterocytes, in the basolateral intercellular spaces of the absorption epithelium, on the basolateral membranes and in the Golgi apparatus of these cells. These results were interpreted to mean that APF is first either taken up by lumen membranes or synthesized in cells and then secreted into the circulatory system [7]. This would explain the presence of APF in plasma and, in particular, the immunological cross-reactivity observed between serum HDL and 'bile lipoprotein complex' [8]. As determined by ELISA with monoclonal anti-APF serum APF represents 0.6 to 1% of the total protein weight of human serum HDL; this percentage is 0.3 to 0.6% for rat serum HDL (results submitted for publication).

The effect of APF on the fate of intravenously administered [^{14}C]cholesterol associated with liposomes containing APF has been studied in the rat [9]. Cholesterol originating from liposomes with APF is stored in liver in greater quantity, metabolized into bile salts in smaller quantity and secreted in bile much later than cholesterol issued from liposomes without APF. Thus, APF seems to play a role in the control of

Abbreviations: APF, anionic polypeptidic fraction; HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); TCA, trichloroacetic acid.

Correspondence: J.C. Hauton, INSERM Unité 130, 18 avenue Mozart, 13009 Marseille, France.

bile-destined cholesterol. A simple hypothesis that might explain this finding is that APF acted on the uptake of cholesterol by hepatocytes through a process mediated by APF-binding sites. The study described herein was undertaken to gain insight in the function of APF by attempting to detect and characterize APF binding sites in plasma membranes and hepatocytes isolated from rat liver.

Materials and Methods

Isolation of human HDL_3 and LDL

Human HDL_3 ($1.125 < d < 1.21$ g/ml) and LDL ($1.006 < d < 1.063$) were isolated by differential ultracentrifugation according to established procedures [10].

Preparation of iodine-labeled APF

APF associated with phospholipids were obtained by zonal ultracentrifugation of human vesicular bile according to the method previously described [6]. APF was labeled with ^{125}I by the iodogen procedure [11]: a 200 µl aliquot of a dichloromethane solution containing 100 µg of 1,3,4,5-tetrachloro-3α,6α-diphenylglycoluril (iodogen from Sigma, St. Louis, MO) was placed in the bottom of a polypropylene tube and left at room temperature under nitrogen until the dichloromethane evaporated to dryness leaving a thin film of iodogen. To this tube were added 1 mg of APF in 0.3 M sodium borate buffer (pH 9.9) and 1 mCi of $Na^{125}I$ (Amersham, RS: 100 mCi/ml). After 15 min at room temperature with gentle stirring, the reaction was terminated by decanting the mixture from the residual iodogen. To ensure safe handling, carrier NaI was added to the iodination mixture until a final concentration of 0.25 M was obtained. Next the mixture was dialysed against several changes of 0.02 M sodium phosphate buffer (pH 7.4) at 4°C for two days. Specific activity ranged from 100 to 200 cpm/ng protein. After extraction with chloroform-methanol according to the method of Folch et al. [12], only 5.6% of the ^{125}I was found attached to the lipids associated to APF. One aliquot of iodinated APF was chromatographed on a Sephadex G-25 (Pharmacia) column (10 cm × 1.0 cm) using a 0.05 M sodium phosphate buffer at a rate of about 3 ml/h. The position of labeled protein was determined by the absorbance at 280 nm and by the radioactivity. A comparison of the chromatographic profiles of APF obtained (Fig. 1) before iodination and after iodination indicates that procedure of iodogen iodination caused neither aggregation nor breakdown.

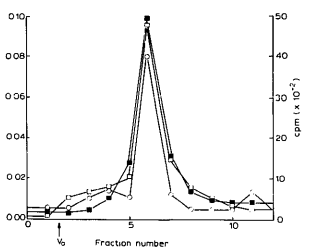

Fig. 1. Comparison of the chromatographic profile on Sephadex G-25 (1 cm × 10 cm) of APF (2.5 mg) before iodination (□——□, $A_{280\ nm}$) and after iodination (■——■, $A_{280\ nm}$; ○——○, cpm). The column was eluted in 0.05 M sodium phosphate (pH 7.4) at 3 ml/h; 0.5 ml fractions were collected.

Isolation of rat liver plasma membranes and assay of ^{125}I-labeled APF binding

Sprague-Dawley rats weighing 100–150 g were used. The liver plasma membranes were isolated according to the procedure of Van Amelsvoort et al. [13] modified by Pastor-Anglada et al. [14]. Purity of the liver plasma membrane preparation was monitored by assay of 5'-nucleotidase according to the procedure of Pastor-Anglada [14]. In the used preparation, 10-fold enrichment in activity was obtained (Spec. act. 5.65 U/mg protein vs. 0.67 U/mg protein in homogenate). The membranes used for binding studies were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.5 mM $CaCl_2$ (working buffer) at a protein concentration of about 2.5 mg protein/ml as determined by the method of Lowry et al. [15].

Binding of ^{125}I-labeled APF to rat liver plasma membranes was achieved by incubating labeled APF with the membranes in 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.5 mM $CaCl_2$ in a final volume of 0.2 ml. In binding assays about 100 µg liver membrane protein and indicated concentrations of labeled APF were used. After incubation for the indicated times, the reaction mixture was cooled in ice for 5 min and then the bound APF was separated from the unbound APF by centrifugation in a Beckman 42.2 Ti rotor at 30000 rpm for 15 min at 4°C. The supernatant containing unbound labeled APF was carefully removed by aspiration. The membrane pellet containing ^{125}I-labeled APF was washed once with 0.2 ml of buffer without dispersing the membranes and the tube was centrifuged again. Radioactivity of the tube containing the membrane pellet was determined in a Kontron gamma scintillation spectrometer with a ^{125}I counting efficiency of 66%. Non specific binding of ^{125}I-labeled APF to the membranes was ascertained by perform parallel assays with a 100-fold excess of unlabeled APF. The difference between the ^{125}I-labeled APF bound to the membranes in the absence and presence of excess unlabeled APF is considered as the amount of ^{125}I-labeled APF bound specifically to the membranes. In

the corresponding blanks, APF was incubated in the absence of membrane.

Isolation of rat hepatocytes and measurement of APF degradation and binding by cells

Hepatocytes of Sprague-Dawley rats were isolated according to Durrington et al. [16] and plated in fibronectin-coated dishes (35 mm) at $2 \cdot 10^6$ cells/dish in 2 ml of Dulbecco's modified Eagle medium (DME) containing 25 mM glucose as penicillin and streptomycin at a concentration of 50000 U/l. Cells were incubated at 37°C for at least 5 h then washed once with medium.

Assessment of APF degradation was done as follows. One ml of fresh DME medium and ^{125}I-labeled APF were added to each dish at the start of the experiment. After incubation at 37°C for the indicated times, dishes were placed on ice and the medium was removed and treated as follows to determine the amount of degraded material released from the cells into the medium. To precipitate protein, cold trichloroacetic acid (TCA) was added to a final concentration of 0.6 M and the mixture was centrifuged at $6000 \times g$ for 10 min. The supernatant was treated with silver nitrate (final concentration 0.05 M) to remove free iodide. The amount of ^{125}I radioactivity found in the non iodide-TCA soluble fraction is referred to as cell-mediated ^{125}I-labeled APF degradation. Non specific degradation was measured in parallel incubations without cells and was used to correct those with cells.

Cells were scraped from dishes and washed three times with 0.15 M NaCl (pH 7.4), and were sedimented by centrifugation at $2000 \times g$ for 10 min after each washing procedure. They were resuspended in a known volume of 0.02 M sodium phosphate buffer (pH 7.4) from which aliquots were taken for protein determination by the method of Lowry et al. [15]. In some experiments, the cells were denatured with 0.6 M TCA. The TCA-insoluble residues were rinsed with 0.6 M TCA, and their radioactivity determined. The latter were taken to represent cell-associated ^{125}I-labeled APF and are referred to as bound plus internalized APF.

For measurement of APF binding, 1 ml of fresh DME medium and ^{125}I-labeled APF were added to dishes at the start of the experiment. Cells were held overnight on ice in a 4°C cold room to prevent internalization then were collected as described above. The final cell pellet was radioassayed in order to determine binding of ^{125}I-labeled APF.

Results

Binding of ^{125}I-labeled APF to rat liver plasma membranes

Binding of ^{125}I-labeled APF to the plasma membranes was dependent on the incubation time. Maximum binding occurred within 60 min (Fig. 2). When isolated rat liver plasma membranes at a fixed concentration were incubated with increasing concentrations of human ^{125}I-labeled APF, a two-component binding curve (Fig. 3A (a)) was obtained. The high-affinity component showed evidence of saturation at about 12.5 μg ^{125}I-labeled APF/ml whereas the other component at higher ^{125}I-labeled APF concentration seemed to be unsaturable. The high-affinity component of the binding curve was abolished when binding of ^{125}I-labeled APF to membranes was carried out in the presence of excess (100-fold) of unlabeled APF (Fig. 3A(b)). The difference between the amount of ^{125}I-labeled APF bound to the membranes in the absence and the presence of excess unlabeled APF was thus an indication of the amount of ^{125}I-labeled APF bound specifically to the high-affinity, saturable binding sites in the membrane (Fig. 3A(c)). Scatchard analysis [17] of this high-affinity, saturable binding curve yielded a linear plot (fig. 3B) suggesting the presence of a single class of independent binding sites for APF on the membranes. From the Scatchard plot, an equilibrium dissociation constant, K_d, of 3.0 μg APF/ml and a maximum binding capacity, B_{max}, of 2.9 μg APF/mg rat liver membrane protein were obtained.

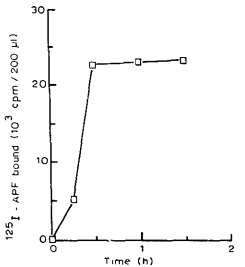

Fig. 2. Effect of time on the binding of human ^{125}I-labeled APF (^{125}I-APF) to rat liver plasma membranes. Binding assays contained 100 μg liver membrane protein and 2 μg labeled APF in 0.2 ml of incubation medium. After incubation (at room temperature) at indicated times, the amount of ^{125}I-labeled APF bound to the membranes was determined as described in Materials and Methods. Each point represents the mean data of duplicate experiments.

The specificity of rat liver plasma membrane ^{125}I-labeled APF-binding sites was studied by competitive binding experiments. Fig. 4 shows the effects of adding increasing concentrations of human APF, HLD_3 and LDL on binding of ^{125}I-labeled APF to the membranes. Much stronger and more specific competition was evident for human APF: 125 μg/ml of unlabeled human APF displaced 50% of ^{125}I-labeled APF. By

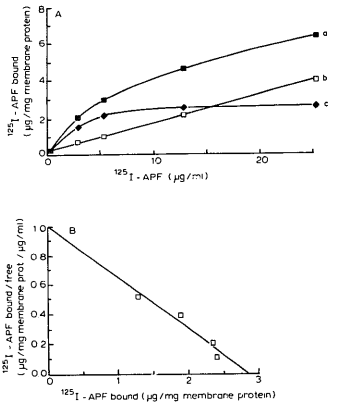

Fig. 3. (A) ^{125}I-labeled APF-binding to rat liver plasma membranes as a function of ^{125}I-labeled APF (^{125}I-APF) concentration. Aliquots of liver membranes (100 μg protein) were incubated with the indicated concentrations of ^{125}I-labeled APF in 0.2 ml of incubation medium, for 1 h at room temperature in the absence (a) and presence (b) of 100-times excess of unlabeled APF. After incubation, the amount of ^{125}I-labeled APF bound to the membranes was determined as described in Materials and Methods. The amount of ^{125}I-labeled APF bound specifically to the membranes (c) was obtained by subtracting the ^{125}I-labeled APF bound in the presence of excess unlabeled APF from that bound in the absence of unlabeled APF. Each point represents the mean data of duplicate experiments, except points of nonspecific binding (single experiment). (B) Scatchard analysis of the data presented in A.

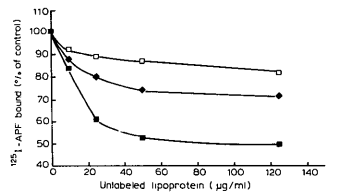

Fig. 4. Effects of human APF, HDL_3 and LDL on the binding of human ^{125}I-labeled APF (^{125}I-APF) to rat liver plasma membranes. Each tube contained 100 μg membrane protein, 10 μg/ml of ^{125}I-labeled APF and the indicated concentrations of either human APF (■), human HDL (◆) and human LDL (□). After incubation at room temperature for 1 h, the amount of ^{125}I-labeled APF bound to the membranes was determined.

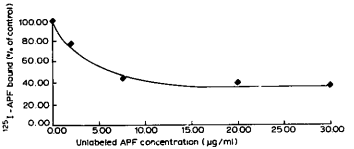

Fig. 5. Effect of unlabeled APF on the binding of ^{125}I-labeled APF on cultured rat hepatocytes. Binding assays were carried out at 4°C overnight with 1 μg of labeled APF and the indicated concentrations of unlabeled APF. The amount of ^{125}I-APF bound on cells was determined as described in Materials and Methods. Each point represents the mean data of triplicate experiments. The '100% control value' for ^{125}I-APF bound in the absence of unlabeled APF was 14.7 ng/mg cell protein.

contrast, human LDL displaced only 18% of ^{125}I-labeled APF in the presence of 125 μg/ml of unlabeled human LDL. With human HDL_3 the degree of displacement was greater than with human LDL: 25% of ^{125}I-labeled APF in the presence of 125 μg/ml of unlabeled human HDL_3.

Metabolism of ^{125}I-labeled APF by rat hepatocytes

When rat hepatocytes were incubated with trace concentrations of ^{125}I-labeled APF at 4°C, a temperature

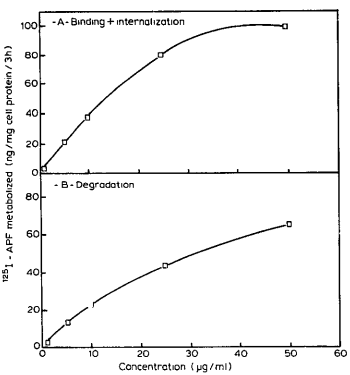

Fig. 6. Concentration dependent binding plus internalization (A) and degradation (B) of ^{125}I-labeled APF by cultured rat hepatocytes. Cells were incubated for 3 h in medium containing the indicated concentrations of ^{125}I-APF. The amount of ^{125}I-labeled APF bound plus internalized and degraded by cells was determined as described in Materials and Methods. Each point represents the mean data of triplicate experiments.

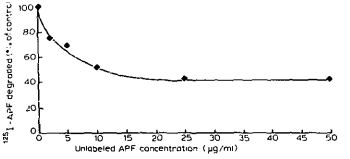

Fig. 7. Effect of unlabeled APF on the degradation of ^{125}I-labeled APF by cultured rat hepatocytes. The effect of unlabeled APF on the degradation of ^{125}I-labeled APF over 3 h was determined in incubations containing a constant low concentration of ^{125}I-APF (1 μg/ml) and increasing concentrations of unlabeled APF. The amount of ^{125}I-labeled APF degraded by cells was determined as described in Materials and Methods. Each point represents the mean data of triplicate experiments. The '100% control value' for ^{125}I-APF degraded in the absence of unlabeled APF was 2.8 ng/mg of cell protein per 3 h.

shown to prevent endocytosis of the ligand [18], significant binding of radioactivity to the cells was observed (^{125}I-labeled APF specific binding was calculated by substrating 'non specific' binding from total binding). The ability of unlabeled APF to compete with ^{125}I-labeled APF for binding sites on rat hepatocytes was studied (Fig. 5). Addition of unlabeled APF led to a competitive reduction in the binding of ^{125}I-labeled APF: 64% at the highest APF concentration tested (30 μg/ml). The uptake and degradation of ^{125}I-labeled APF was examined as a function of ligand concentration. An incubation time of three hours was selected for this study since at that time cell-associated radioactivity had reached a steady-state level while degradation still continued linearly (data not shown). Figs. 6A and 6B show that cultured hepatocytes are able to take up and degrade APF upon incubation at 37°C. Uptake and degradation was directly demonstrated to increase with increasing ligand concentration in a linear fashion up to the concentration in the range of 10 μg/ml after which it appeared to be saturable. The ability of unlabeled APF to compete with ^{125}I-labeled APF for degradation is shown in Fig. 7. Addition of unlabeled APF led to a competitive reduction in the rate of degradation of ^{125}I-labeled APF: 60% at the highest APF concentration tested (50 μg/ml).

Discussion

The results of this study show that radioiodinated APF was bound by rat liver plasma membrane in a concentration-dependent and apparently saturable process. Scatchard analysis of the binding data yielded a straight line plot, suggesting that ^{125}I-labeled APF binds to a single class of non-interacting binding sites. Experiments also showed that rat hepatocytes bound, internalized and degraded APF by a saturable process. The results of hepatocyte experiments confirmed those using liver membranes and suggested that the APF binding sites observed in isolated liver membranes are not artificially generated during the isolation.

The liver plays an important role in lipoprotein catabolism. High-affinity saturable binding sites for HDL have been detected in rat hepatocytes [19–21] and rat liver membranes [22]. A high-affinity receptor for LDL has been found on the surface of cells in various organs [23], in particular the liver. In view of these findings, we have studied in the present report the specificity of rat liver plasma membrane binding sites for ^{125}I-labeled APF by competitive binding experiments with LDL and HDL. The resulting data showed that more effective competition was evident for unlabeled human APF. Unlabeled LDL was unable to compete for binding of ^{125}I-labeled APF. This suggests that APF binding to liver membranes involves specific binding sites different from those of LDL. Addition of unlabeled HDL_3 led to a competitive reduction in the binding of labeled APF at a level between that obtained with unlabeled APF and with unlabeled LDL. One explanation for this result would be the presence of APF detected in HDL_3 by ELISA assay, using an anti-APF monoclonal serum. The presence of APF as protein constituent would account for the observation of partial competition by human HDL_3 for binding of ^{125}I-labeled APF. Results showed the presence of four specific epitopes of APF exposed on the surface of HDL. These data suggested that APF is a distinct minor structural entity in HDL and can explain the observed competition (these results are submitted for publication).

In conclusion, the present study showed that APF binds to liver plasma membrane and is degraded by rat hepatocytes through a saturable process. Plasma membranes from rat liver contain high affinity, saturable binding sites for ^{125}I-labeled APF. These APF binding sites seem to be specific for APF, properties typical of a ligand–receptor interaction. The specificity, the nature and the possible regulation of these APF-binding sites remain to be fully elucidated. Likewise, the biological significance of the high-affinity APF-binding sites remains unclear, although results obtained in vivo [9] seemed to implicate this protein in the control of bile-destined cholesterol. Also, we postulate that this protein is involved in the recognition of cholesterol by hepatocytes. This hypothesis would explain the quantitative importance of the role of HDL cholesterol in the secretion of biliary cholesterol and bile salts.

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